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13. ABSTRACT (Maximum 200 Words)

The small GTP-binding protein Rac controls essential functions, including actin cytoskeleton reorganization, cell proliferation, cell cycle progression, adhesion, migration and invasion. The relationship of Rac to prostate carcinogenesis has not been extensively studied. However upstream activators of Rac have been described to be hyperactivated in prostate cancer, and it is well known that growth factors are very important in the control of prostate cancer proliferation and progression, as well as in the maintenance of growth during androgen independency. Chimaerins, through their Rac-GAP activity, accelerate the hydrolysis of GTP from Rac, leading to its inactivation. To date four chimaerin isoforms have been isolated and reported: α1, α2-, β1- and β2-Chimaerin. While α1- and β1-chimaerin are restricted to brain and testis, respectively, α2- and β2-chimaerin are widely expressed. No experimental information has been reported about the possible role of chimaerins in prostate cancer. Likewise, there are no information available about the expression of different chimaerin in prostate cancer cell lines. Our work hypothesis is that by inhibiting Rac function in prostate cancer cells, chimaerins will impair proliferation and reduce the invasive properties of prostate cancer cells.

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INTRODUCTION

The small GTP-binding protein Rac controls essential functions, including actin cytoskeleton reorganization, cell proliferation, cell cycle progression, adhesion, migration and invasion. Whereas the relationship of Rac to prostate carcinogenesis has not been extensively studied, several papers have described that upstream activators of Rac are hyperactivated in prostate cancer, and it is well known that growth factors are very important in the control of prostate cancer proliferation and progression, as well as in the maintenance of growth during androgen independency. Rac belongs to the Rho family of small GTP-binding proteins, and cycles between an "on" (GTP-bound) and an "off" (GDP-bound) state, steps that involve guanine nucleotide exchange factors (GEFs) and GTPase activating proteins, which accelerate GTP hydrolysis, respectively (1-3)

Chimaerins, through their Rac-GAP activity, accelerate the hydrolysis of GTP from Rac1, leading to its inactivation. To date four chimaerin isoforms have been isolated: α1, α2-, β1- and β2-Chimaerin. While α1- and β1-chimaerin are restricted to brain and testis, respectively, α2- and β2-chimaerin are widely expressed (4,5). However, there are no reported information or experimental data describing chimaerin activity and/or expression in prostate cancer cells. Since chimaerins inhibit Rac function, it is predictable that they will have profound effects on Rac mediated signaling, and therefore impact on proliferation and invasion in prostate cancer cells.

BODY

Characterization of chimaerin isozymes in prostate cancer cells.

To determine which chimaerin isoforms are expressed in prostate cells, we initiated studies to determine their expression using non-quantitative RT-PCR. We used specific primers for the different chimaerin isoforms and GADPH based on the reported human sequences. We also initiated Western blot assays. We used LNCaP and C4 cells as androgen-dependent model with different sensitivities to androgens. The PC3 cell line was used in our experiments as a model for as an androgen-independent cells. Our experimental data using non-quantitative RT-PCR (Figure 1) showed that LNCaP cells do not express α2- or β2-chimaerin. C4 cells express only β2-chimaerin at very low levels and PC3 cells express α2- and β2-chimaerin at similar levels.

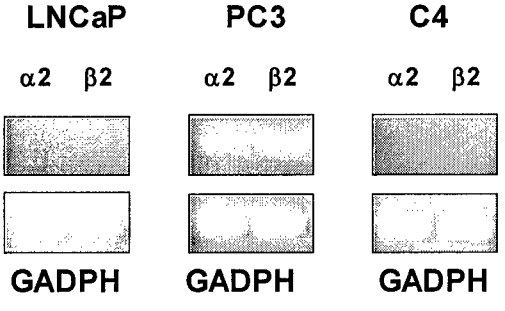

Figure1. Non-quantitative RT-PCR of α2-, β2-chimaerin and GADPH in LNCaP, PC3 and C4 cells.

Since we have recently determined that our β2-chimaerin antibody (rat monoclonal) cross-reacts with α2-chimaerin (Figure 2), and both isoforms have the same molecular size, we designed a peptide based on the α2-chimaerin sequence. This peptide was inoculated into rabbits. We are currently determining whether the antibody indeed detects α2-chimaerin and hopefully should not cross-react with β2-chimaerin, as a specific peptide was indeed designed for these experiments.

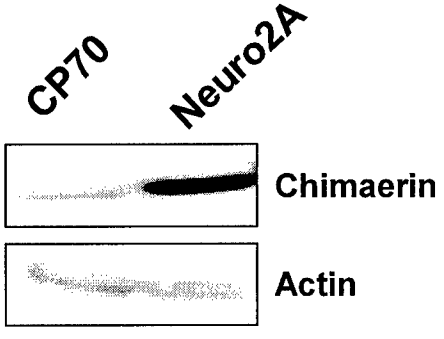

Figure 2. Rat chimaerin antibody used in western blot analysis for the detection of cross-reacts with α2-chimaerin. Cell lysates from the human ovarian cancer cell line CP70, that express α2- but not β2-chimaerin and murine Neuro 2A cell line, that express both isoforms (Kazanietz lab, unpublished data), were analyzed for chimaerin expresion.

Cloning of α2 chimaerin.

As a cDNA for the α2-chimaerin isoform was not available in our laboratory, and this would be needed not only for functional experiments as well as for the characterization of the antibody, we decided to clone this cDNA using RT-PCR. We used cDNA generated from fresh PC3 mRNA and by RT-PCR was carried out specific primers. Restriction sites have been included in order to facilitate subcloning into various expression vectors. We have been successful in cloning this chimaerin isoform and then we subcloned it into TOPO®- vector (Invitrogen). The identity of the cDNA was verified by sequencing. Results revealed 100% identity with the sequence reported in NCBI databases, suggesting that no mutations were introduced in the PCR reaction.

Generation of chimaerin adenoviruses.

The generation of recombinant adenoviruses to study the role of specific chimaerins represents an essential step, as we have developed conditions in our laboratory to achieve nearly ~100% of the prostate cancer cells expressing recombinant proteins. We normally use the AdEasyR system (Stratagene, La Jolla, CA) to generate adenoviruses. The multicloning site of this vector was previously modified in our laboratory to facilitate easy subcloning, and a N-terminal HA-tag has been included for easy detection. Previously, in our laboratory we have successfully generated adenoviruses for *wild type* β2- chimaerin (β2-chim-AdV) and the β2-chimaerin GAP domain (β-GAP-AdV).

I have been actively involved in the generation of other adenoviruses that would be needed in our experiments, as follows:

β2 chimaerin

AdVs for GAP inactive mutants: **β2-chim-ΔEIE-AdV** and **β2-chim-298RRR-AdV.**
AdVs for phorbol ester/DAG unresponsive chimaerins: **β2-chim-C246A-AdV.**
AdVs for SH2 domain : **β2-chim-ΔSH2-AdV.**

The β2-chimaerin crystal structure of β2-chimaerin has been recently reported, as part of a collaborative effort between our laboratory and thelaboratory of Dr. Jim Hurley at the NIH (6). In this study it was found that several β2-chimaerin mutants present high sensitivity to phorbol ester-induced translocation as well as enhanced RacGAP activity *in vivo*. Due to this important finding we decided to incorporate these mutants to our study, as they will greatly contribute to our functional studies. Thus, I have been actively involved in the generation of adenoviruses encoding for two active mutants: **β2-chimaerin Q32A** and **β2-chimaerin -I130A**

α 2 chimaerin

We are currently generating the adenoviral construct for the generation of an adenovirus for *wild type* α2 *chimaerin*: **α2-chim-AdV.** Adenoviruses for active and inactive mutants are in the process of being generated.

Expression of chimaerins upon adenoviral infection.

Since all adenoviruses constructs have been designed with an epitope tag (HA-tag), we carried out infections in prostate cancer cells and determined whether they can be detected using an anti-HA antibody. Experimental data showed that all adenoviruses work successfully upon infection of prostate cancer cells, as well as in other cellular models. Chimaerin expression was detected 24 hours after infection. Although a careful time-course analysis has not been carried out, it is expected that expression should last for several days. This assumption is based on similar experiments that we have carried out using adenoviruses with the same adenoviral expression vector.

Cloning of a putative β2-chimaerin promoter.

Transcription in eukaryotic cells is regulated at multiple levels. It has been determined that the methylation status of CpG islands in the gene directly affects the DNA-protein interactions. Most of the CpG islands in the promoter remain unmethylated, but when methylation occurs the associated gene is silenced. Thus, DNA methylation acts as a major epigenetic modification to maintain stable gene silencing. Aberrant DNA methylation impacts on gene transcription and it has been linked to cancer formation (7,8). Since several prostate cancer cell lines have low chimaerin expression, we sought to investigate whether the effect was at the promoter level. To date, there is no information on the promoter for β2 chimaerin. In our laboratory we detected *in silico* a region in the β2-chimaerin locus with promoter characteristics. This region contains a putative CpG island. A 1 Kb genomic DNA fragment 5′ upstream from the β2-chimaerin start codon was cloned by PCR into TOPO®- vector (Invitrogen).

Using PCR we have amplified and subcloned different fragments (see list above) of the 1 Kb genomic segment into plasmid pRTK-Luc (this is a construct that encodes for a luciferase gene under the control of a TK promoter). The constructs generated were as follows: **pR-81-Luc**, **pR-181-Luc**, **pR-281-Luc**, **pR-381-Luc**, **pR-481-Luc**, **pR-581-Luc**, **pR-1000-Luc** (the number represent the position of the first nucleotide upstream of the ATG). **pR-BASIC** (luciferase with no promoter) as a negative control and **pRTK-Luc** as a positive control.

We have transiently co-tranfected these constructs together with a Renilla expression vector (for normalization) into several cell lines. Experimental data revealed that all constructs induce luciferase expression upon transfection (Figure 3). We are currently analyzing in a comparative manner the various constructs upon transfection into prostate cancer cells (as well as in other cell types).

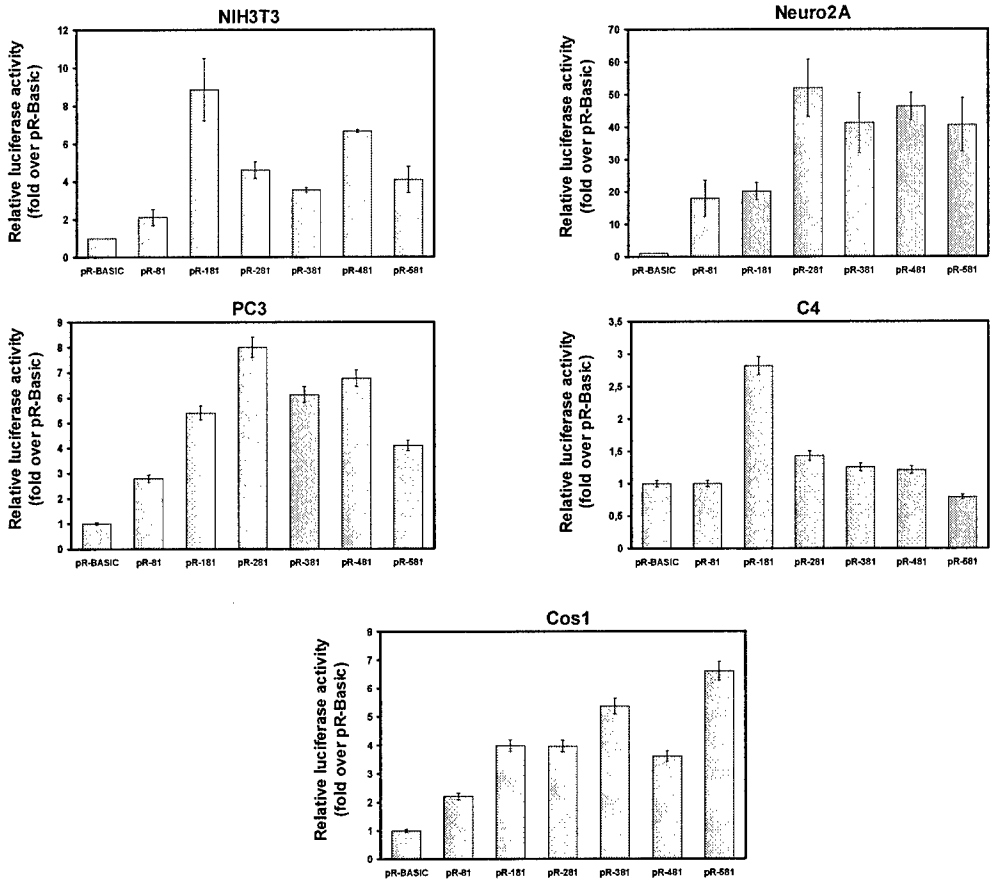

Figure 3. Luciferase activity of different constructs of β2-chimaerin putative promoter in several cell lines. NIH3T3, Neuro2A, PC3, C4 and Cos1 cells were transfected with 0.5μg of each reporter construct, and 24 h after transfection luciferase activity of cell lysates was determined. Values represent the means ± S.D.from trhee different experiments.

In a different set of experiments we have focused on the methylation of the promoter. The constructs described above were methylated *in vitro* by using the enzymes SssI methylase, HpaII methylase and HhaI methylase. The methylation status was determined by digestion with the respectives enzymes and visualization in a agarose gel (Figure 4a). In a preliminary experiment we observed that upon transfection of the methylated constructs no luciferase activity can be detected (Figure 4b). These results suggest that the1 Kb fragment that we have isolated is indeed susceptible to methylation and therefore one may expect that methylation may greatly influence the expression of the gene in certain cancer cells, including prostate cancer cells.

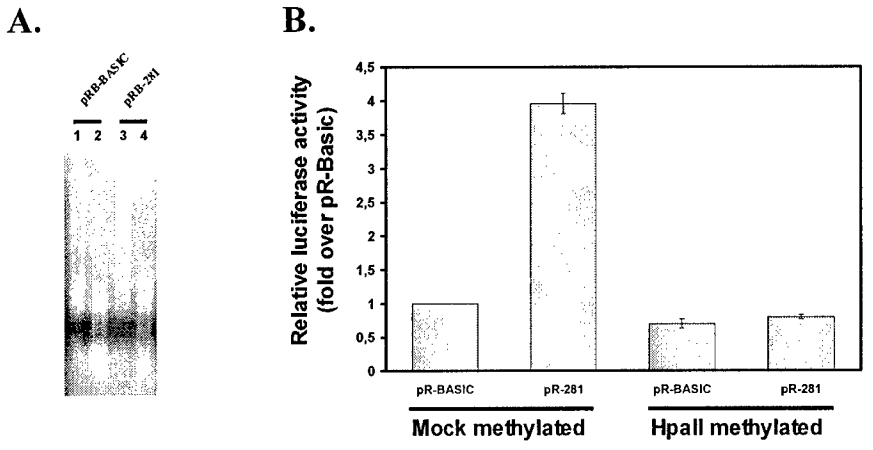

Figure 4. Effect of in vitro methylation on β2-chimaerin putative promoter activity. A, pR-BASIC and pRB-281 were methylated *in vitro* with HpaII methylase. The extent of methylation was assessed by comparing digestion patterns of unmethylated (lanes 2-4) and methylated (lanes 1-3) constructs with HpaII. **B**, methylated and unmethylated pR-BASIC and pR-281 constructs were transfected into Cos1 cells and assayed for luciferase activity. Values represent the means ± S.D.from trhee different experiments.

Future directions.

The next goals will be:

1. To assess weather chimaerins regulates mitogenic signaling in prostate cancer cells.
2. To determine how chimaerins affect to cytoskeleton reorganization, migration and invasion in prostate cancer cells. The adenoviruses for different chimaerin isoforms and mutants will be used in these experiments.
3. To continue the characterization of the putative β2-chimaerin promoter and to determine whether methylation is a critical factor in β2 chimaerin expression.

KEY RESEARCH ACCOMPLISHEMENTS

1. We have initiated the characterization of chimaerin isoform expression in prostate cancer cell lines.
2. We generated adenoviruses or adenoviral constructs that are essential for functional studies in prostate cancer cells.
3. We clone a 1 kB fragment that corresponds to the β2-chimaerin promoter.
4. We generated reporter constructs for different regions of the β2-chimaerin promoter, which will be used in luciferase assays.
5. We initiated studies aimed at determining whether the β2-chimaerin promoter is regulated by methylation.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

In the last year we initiated experiments to characterize the regulation and function of β2-chimaerin and related chimaerin isoforms in prostate cancer cells. This protein regulates the function of the small GTP-binding protein Rac, which plays essential roles in mitogenesis, transformation, and the metastatic cascade. We have obtained preliminary data regarding the expression of chimaerin isozymes in prostate cancer cell lines. It seems that expression is low in prostate cancer cells, which is in agreement with data in other types of cancers, such ad breast cancer cells (unpublished observations from our laboratory). Therefore, understanding the events that control the expression of the β2-chimaerin gene is very important. The cloning of a putative promoter and its characterization will further enhance the understanding of the regulation of this molecule. In addition, in the present year we have successfully generated an important set of tools, in addition to those already available in the laboratory, such adenoviruses for different chimaerins isoforms and mutants. We have successfully assessed and validated experimentally all these tools.

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APPENDICES

None.

ABBREVIATIONS

AdV: adenovirus
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
DAG: diacylglycerol
GAP: GTPase activating protein
Kb: Kilobase
MOI: Multiplicity of infection
NCBI: National Center for Biotechnology Information
PKC: protein kinase C
PMA: Phorbol 12-myristate 13-acetate
PCR: Polymerase Chain Reaction
Rac-GAP: Rac-GTPase activating protein
RT: Reverse Transcription
TK: Thymidine Kinase